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Preparation of CHO cell-derived rhIFN-ω-Fc with improved pharmacokinetics

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ABSTRACT

Interferon-omega (IFN-ω) may be a useful, promising and alternative antiviral agent, in addition to IFN-α-2a and IFN-α-2b. To improve the pharmacokinetics of IFN-ω for clinical use, the recombinant human IFN-ω-Fc fusion protein (rhIFN-ω-Fc) was expressed in a Chinese hamster ovary cell line (CHO-S), due to the longer serum half-life of rhIFN-ω-Fc compared to the native IFN-ω protein, and purified by affinity chromatography. Physicochemical characterization of the purified fusion protein was performed by SDS–PAGE electrophoresis, dot blot analysis and N-terminal amino acid sequence analysis. The results show that rhIFN-ω-Fc was highly expressed at the predicted size and with the N-terminal amino acid sequence. The antiviral activity was determined by the ability of IFNs to inhibit the cytopathic effects (CPEs) of vesicular stomatitis virus (VSV) on the human amnion WISH cells. The rhIFN-ω-Fc expressed in CHO-S cells has a specific activity of 1.6×10^7 IU/mg compared to rhIFN-ω expressed in yeast, which has a specific activity of 7×10^7 IU/mg. Equimolar concentrations of rhFN-ω and rhIFN-ω-Fc were administered to rabbits for pharmacokinetics comparison. The terminal half-life of rhIFN-ω-Fc was 35 times higher than that of rhIFN-ω. Thus, rhIFN-ω-Fc can be used as a prospective antiviral candidate especially for the treatment of chronic viral disease, such as hepatitis C virus (HCV) infection.

1. Introduction

Interferons (IFNs) are a family of related cytokines that are classified into subgroups, type I and type II, according to receptor specificity and sequence homology. Type I interferons consist of multiple IFN-subtypes, IFN-α, IFN-β, IFN-ω, and IFN-ι. In contrast, IFN-γ is the only type II IFN (Pestka et al., 2004). Type I IFNs represent the first line of defense against many types of viral infection (Crance et al., 2003; Briolant et al., 2004; Julander et al., 2007; Chang and George, 2007). Currently, combination therapy of IFN-α and ribavirin is the most effective treatment and the standard for care for HCV infection (Stauber and Stadlbauer, 2006; Michaels and Nelson, 2010).

Human interferon-ω (hIFN-ω) was originally reported in 1985 (Hauptmann and Swetly, 1985; Capon et al., 1985) and shown to be antigenically different from human IFN-α, IFN-β and IFN-γ (Adolf, 1987). Human IFN-ω is a glycoprotein, molecular weight 22 kDa, produced mainly by adult human lymphocytes (Adolf, 1987; Adolf et al., 1990). Human IFN-ω is a 174 amino acid, single polypeptide chain containing two disulfide bonds and a potential N-linked (Asn−) site at amino acid 80 (Adolf et al., 1990). Human IFN-ω has 65% amino acid sequence homology and similar function as interferon-α (IFN-α). Human IFN-ω can still affect patients who are resistant to the interferon-α because of the different antigenicity and immunogenicity between interferon-ω and IFN-α. Fully glycosylated recombinant human IFN-ω produced from a Chinese hamster ovary cell line, CHO-S (glycosylated IFN-ω), induced a sustained viral response (SVR) in patients infected with HCV genotypes 1–3 (Buckwold et al., 2006, 2007).

In addition to the effects of IFNs, one main problem is the poor pharmacokinetics of recombinant human interferon (rhIFN-ω). The treatment period for chronic viral disease, such as HCV infection, is one and a half years. Therefore, improving the serum half-life is an important medical and commercial goal. In general, there are two approaches for improving serum half-life. PEGylation is one method used for improving IFN-α-2a and IFN-α-2b serum half-life as both IFNs were approved for treatment of HCV infection (trade name PEGASYS (peginterferon alfa-2a) and PegIntron® (Peginterferon alfa-2b)) (Glue et al., 2000; Perry and Jarvis, 2001; Berenguer et al., 2009). PEGylation is a complicated process because the recombinant protein must be purified, then modified, and finally isolated from the native protein.

When compared to PEGylation, the production of human serum albumin (HSA) (Osborn et al., 2002; Zeuzem et al., 2008; Rustgi, 2009) or human IgG1 Fc (Jones et al., 2004; Okuse et al., 2005;

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Schrieb et al., 2006; Dumont et al., 2006; Jazayeri and Carroll, 2008; Huang, 2009) fusion proteins is a simple and effective method for prolonging serum half-life. Glycosylated IFN-ω was found to be the most potent antiviral agent of all the IFNs tested including human IFN-α, -β, -γ and non-glycosylated IFN-ω expressed in *Escherichia coli* (Buckwold et al., 2006). Considering hIFN-ω is a glycoprotein, yeast has different glycosylation patterns compared to the mammalian cell. Preparation of rhIFN-ω-Fc fusion protein in mammalian cells, such as CHO-S, PERC.6, NSO, and BHK21, is the best choice for improving the serum half-life with correct glycosylation. In our present study, the IFN-ω-Fc was expressed in CHO-S cells and further purified. Physicochemical characterization, antiviral activity and pharmacokinetics of the purified rhIFN-ω-Fc were analyzed further.

2. Materials and methods

2.1. Materials

E. coli DH5α competent cells were purchased from the Tiangen Biological Company. The expression vector pMH3S and the Chinese hamster ovary cell line (CHO-S) were grown and maintained in our laboratory. The recombinant expression plasmid pHF including the human IgG1 subclass C region gene and the pMD-IFN-ω plasmid containing IFN-ω cDNA were kept in our laboratory DNA endonuclease, *T4* DNA ligase, Ex Taq and pMD-T vector were purchased from the TaKaRa Company. The *E. coli* plasmid extraction kit was purchased from OMEGA. Anti-human IgG (Fc-specific) and peroxidase conjugated anti-human IgG (Fc-specific) were purchased from Sigma. Dulbecco's modified Eagle's medium DMEM/HAM's F-12 and fetal bovine serum (FBS) were purchased from Sigma.

2.2. Construction of expression vectors with different signal peptides

The plasmid pMD-ifn-ω containing IFN-ω cDNA and the plasmid pHF including human IgG1 subclass C region gene were used as sources for template DNA. PCR was performed using the following primers containing ifn-ω-up: 5′-AAT**GAATTC** TCTGGGCTGTGATCTGCCTC-3′; IFN-ω-lower: 5′-AGATGAGCCCAGGTCTCTATC-3′; IFN-ω-Fc: 5′-GATAGAGACCTGGGCTCATCTGTTGAGCCCAAATCTTGTG-3′; and Fc-lower: 5′-AAT**GCGGCCGC**TCATTAACCCGGAGACAGGGAGAG-3′. The IFN-ω-up primer containing an engineered *Eco* RI site (bold, underlined) and the primer Fc-lower containing an engineered *Not* I site (bold, underlined) were used for cloning PCR products into pMH3S. The IFN-ω and Fc genes were amplified using these two primer sets. After denaturation for 5 min at 94 °C, IFN-ω amplification was performed for 25 cycles through a regime of 20 s at 94 °C, 20 s at 56 °C, and 40 s at 72 °C. For the amplification of Fc, the primer pair IFN-ω-Fc/Fc-lower was used. For the amplification of fusion gene IFN-ω-Fc, the primer pair IFN-ω-up/Fc-lower was used. After successful construction of the IFN-ω-Fc fusion gene, a different signaling peptide gene sequence was fused to the N-terminus of the IFN-ω-Fc by overlap extension PCR (OE-PCR). The purified PCR product and vector were digested with *Eco* RI and *Not* I, gel-purified, and ligated. *E. coli* DH5a was chemically transformed with the recombinant vector and cultured at 37 °C in LB containing ampicillin (100 μg/ml) for selection of recombinants. The resulting plasmids were designated pMH3S-IFN-ω-Fc, pMH3-ori-IFN-ω-Fc, pMH3-kappa-IFN-ω-Fc and pMH3-Epo-IFN-ω-Fc. The plasmids contained the tPA leader signal peptide, mouse monoclonal antibody kappa light chain signal peptide, Epo signal peptide, IFN-ω original signal peptide, followed by the human mature IFN-ω coding sequence and the hinge-CH2-CH3 constant region of a human IgG1. Finally, all constructs were confirmed by DNA sequencing analysis.

2.3. Transient expression analysis of constructs with different signal peptides in CHO-K1

To reach 90–95% confluency ($0.5–2.0 \times 10^5$ cells/well/24-well plates) at the time of transfection, the cells were plated in growth medium, without antibiotics, one day before transfection. DNA–Lipofectamine 2000 complexes were prepared by diluting 1 μg pMH3S-IFN-ω-Fc in 50 μL of Iscove's modified Dulbecco's medium (IMDM) without serum; diluting 3 μL Lipofectamine 2000 in 50 μL of IMDM medium without serum; gently mixing and incubating for 5 min. The diluted DNAs were combined with diluted Lipofectamine 2000, mixed gently and incubated for 20 min while DNA–Lipofectamine 2000 complexes formed. To each well, 100 μL of the DNA–Lipofectamine 2000 complex was added. The plates were mixed gently by rocking. The cells were incubated in a incubator at 37 °C with 5% CO_2 for 60–72 h until ready to assay for transient expression. The serially diluted supernatants were transferred to a nitrocellulose membrane and attached by drying. Non-specific binding to the membrane was blocked by incubation for 2 h at room temperature in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 500 g/L defatted milk powder. The blots were washed three times with TBST and then incubated with HRP-conjugated goat anti-human IgG. Finally, the blots were washed four times in TBST. Bound peroxidase was visualized by chemiluminescence (Pierce SuperSignal Kit).

2.4. Screening of high yield clones

CHO-S cells are a clonal isolate derived from Chinese hamster ovary (CHO-K1) cells. The CHO-S cells are adapted to serum-free suspension culture in CD CHO medium supplemented with L-glutamine and HT Supplement for transient or stable expression of recombinant proteins. Chinese hamster ovary cells (CHO-S) were transfected with pMH3S-IFN-ω-Fc. Transfected cells were selected for growth in the presence of 1.6 mg/mL G418. Cell clones were selected and further cultured in suspension in Dulbecco's modified Eagle's medium DMEM/HAM's F-12 (1:1 mixture). A CHO-S clone was selected within two weeks of growth and adapted for further growth in spinner flasks using a production medium developed in-house. Cells were maintained in suspension culture in 300-ml and 5000-ml spinner flasks at 50 rpm on magnetic stir plates at 37 °C. The fusion protein was secreted into the culture supernatant.

2.5. Quantification of the supernatant secreted rhIFN-ω-Fc by ELISA

The rhINF-ω-Fc content in the culture supernatant was quantified by ELISA. Microtiter plates were coated with a goat anti-human IgG (γ-chain specific) antibody (100 μl/well, 0.02 mg/ml, Sigma, Germany) in 0.1 M sodium bicarbonate buffer, pH 9.6 for 12 h at 4 °C. For each assay, we performed a duplicate standard curve by utilizing serial dilutions (1:2) of rhIFN-ω-Fc standard (2.23–0.01 pmol/ml) to quantify sample concentrations. Fifty microliters per well of the standard and sample serial dilution were applied and incubated for 1 h at room temperature. Afterwards, the plates were incubated with a goat anti-human IgG (γ-chain specific) antibody horseradish peroxidase (HRP) conjugate in PBS-T/1% BSA for 1 h at room temperature, followed by staining with O-phenylenediamine dihydrochloride (OPD, Fluka, Switzerland). To each well, 100 μl of staining solution [120 μl OPD (100 mg/ml), 12 ml (0.035 M citric acid monohydrate, 0.067 M sodium phosphate dibasic dihydrate, pH 5.0), 12 μl 35% hydrogen peroxide solution] was added and incubated at room temperature. The enzymatic

reaction was stopped by adding 100 μl 25% sulfuric acid to each well. The optical density was read using an ELISA plate reader at 450 nm.

2.6. Purification of rhIFN-ω-Fc

The secreted supernatant containing the rhIFN-ω-Fc proteins expressed in the CHO-S cells was purified by affinity chromatography via HiTrap™ rProtein A FF (GE Healthcare, Sweden). Cell-free culture supernatant (2200 ml) containing rhIFN-ω-Fc was loaded onto an XK16/20 column including 25-ml rProtein A FF gel at a flow rate of 5 ml/min (150 cm/h). After extensive washing of the column with 20 mM sodium phosphate buffer, pH 7.0, rhIFN-ω-Fc was eluted with 0.1 M glycine–HCl, pH 2.7. The eluted fraction was rapidly neutralized with 1 M Tris, pH 9.0. After elution, the column was regenerated with 0.1 M glycine–HCl, pH 2.7. After the concentrations were determined by the Lowry method, the eluted protein was applied to SDS–PAGE.

2.7. SDS–PAGE and deglycosylation analysis

The purified rhIFN-ω-Fc protein was analyzed by SDS–PAGE electrophoresis in a 12% resolution gel under non-reducing and reducing conditions. Under non-reducing conditions, the sample was diluted 1:2 in 2× sample buffer. Under reducing conditions, the sample was treated with 5% 2-mercaptoethanol (2-ME, Fluka, Switzerland) at 100 °C for 10 min and diluted 1:2 in 2× sample buffer.

For glycosylation analysis under reducing conditions, the purified rhIFN-ω-Fc protein (3.6 nmol/ml) was denatured at 70 °C for 10 min in denaturation buffer (5% SDS, 1% 2-ME, New England BioLabs, UK). PNGase F (from flavobacterium meningosepticum, 500 U, New England BioLabs, Hitchin, UK) digestion was carried out in 0.05 M sodium phosphate buffer, pH 5.5 with 1% (w/v) NP40 for 6 h at 37 °C. The proteins were stained with Coomassie brilliant blue for 30 min followed by destaining in a solution containing 40% ethyl alcohol and 10% acetic acid.

2.8. Antiviral activity of the purified rhIFN-ω-Fc

The antiviral activity was assayed according to the cell pathogenic effect (CPE) of vesicular stomatitis virus (VSV) on WISH cells. WISH cells were cultured in complete medium (MEM with 10% fetal serum). The medium was discarded, and the WISH cells were washed twice with PBS. The cells were collected after trypsinizing and were suspended in complete medium (3×10^5 ml^{-1}). The suspended cells were inoculated in 96-well plates at 100 μl/well. The cells were incubated 4–6 h at 37 °C with 5% CO_2. The serial diluted rhIFN-ω-Fc was added to each well at 100 μl/well and incubated 16–24 h at 37 °C with 5% CO_2. The supernatant was discarded. The VSV was diluted with MEM supplemented with 3% FBS to 100 Cell Culture Infective Dose 50% per ml ($CCID_{50}$/ml), and 100 μl/well was added to the well. The plate was incubated 24 h at 37 °C with 5% CO_2, followed by the addition of 10 μl 5 g/l MTT solution into each well. After incubation for another 4 h at 37 °C, 50 μl of MTT lysing solution (20% SDS, 50% DMF) was added to each well and incubated overnight. The plates were read at 570 nm, and the unit of each sample's antiviral activity was calculated. The value of 1 IU/ml was defined as the appearance of 50% CPE. The specific activity was deduced according to the dilution.

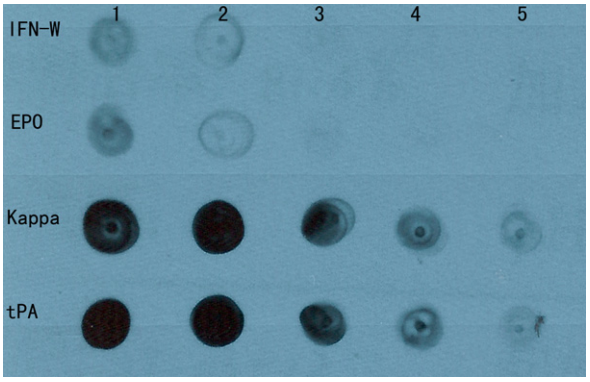

Fig. 1. Transient expression analysis of the different constructs with different signal peptides in CHO-K1 by dot blot analysis. Lanes 1–5 serial dilution of the supernatant after transient transfection from 5 μl to 0.3125 μl.

2.9. Pharmacokinetic studies of the purified rhIFN-ω-Fc

New Zealand White (NZW) rabbits were used in the pharmacokinetic studies. Equimolar amounts of rhIFN-ω (1 nM/kg, 20 μg/kg) and rhIFN-ω-Fc (1 nM/kg, 46 μg/kg) were injected subcutaneously. Blood was sampled via the ear vein at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120 and 144 h after administration. Serum was frozen and analyzed for IFN-ω content using a human IFN-ω ELISA kit (Bender MedSystems™). Pharmacokinetic parameters were analyzed using GraphPad Prism 5.0 (non-linear regression–one phase decay) software.

3. Results

3.1. Construction of expression vector with different signal peptides, expression and purification of rhIFN-ω-Fc

After transfection, the expression levels of the different signal peptide constructs were analyzed by dot blot as shown in Fig. 1. The constructs with tPA and mouse kappa light chain signal peptide displayed higher expression levels compared to the EPO and IFN-ω signal peptides. The tPA signal peptide construct was chosen for stable transfection. A cell line demonstrating the highest expression level was selected and used for large scale culture. The cells cultivated in a 5000-ml spinner flask were harvested after 3–4 days with an average cell density of 8×10^6 cells/ml and cell viability >95%. According to the ELISA analysis, the average volumetric titer was 85 mg/l. The media were centrifuged at 12,000 rpm for 10 min. The supernatants were collected and further purified. The purification was performed by affinity chromatography on Protein A Sepharose FF. On average, 92% of the loaded protein was recovered (Table 1). The purified rhIFN-ω-Fc fusion protein was used for protein characterization without further purification.

3.2. Protein characteristics

Purified rhIFN-ω-Fc was analyzed by SDS–PAGE under reducing and non-reducing conditions. The fusion protein existed in

Table 1
Purification of rhIFN-ω-Fc fusion protein.

	Protein concentration (mg/ml)	Total volume (ml)	Total protein (mg)	Total bioactivity (IU/mg)	Yield (%)	Purity (%)
Crude protein	0.085	2200	187	–	100	–
Protein A Sepharose FF	2.3	75	172.5	3.7 × 107	92	95

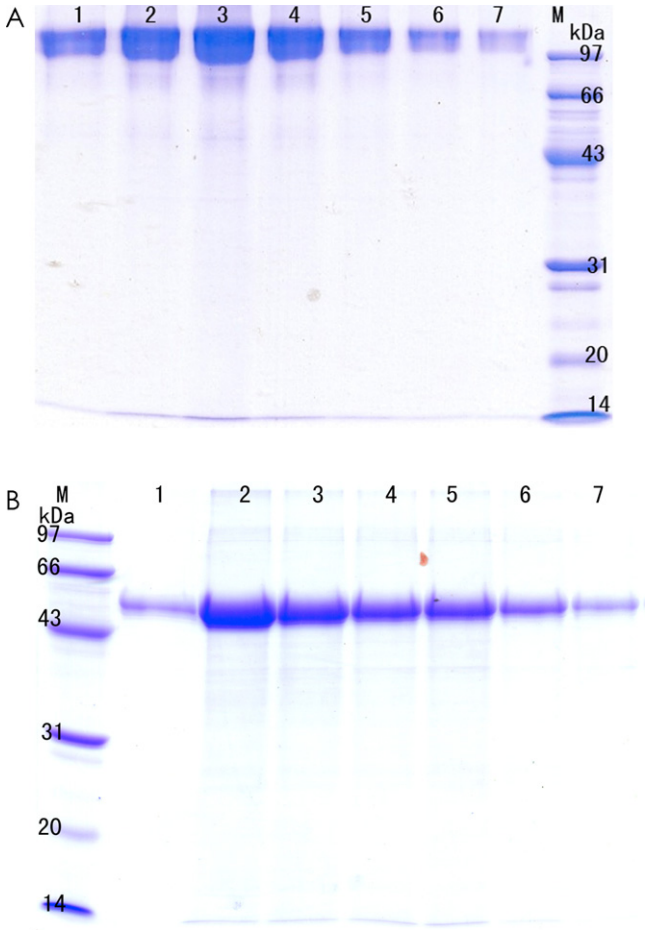

Fig. 2. Separation of rhIFN-ω-Fc by SDS–PAGE followed by Coomassie brilliant blue staining. (A) Non-reducing conditions; (B) reducing conditions. Lane M: low molecular weight marker; lanes 1–7, purified rhIFN-ω-Fc from different fractions of the protein preparation.

a homodimer pattern under non-reducing conditions and in a monomer pattern under reducing conditions (Fig. 2). The result showed that fusion protein formed homodimers consisting of two identical polypeptide chains in CHO-S cells. The fusion protein rhIFN-ω-Fc is comprised of 406 amino acids with a molecular weight of 50 kDa, of which 46 kDa is contributed by the polypeptide backbone. The apparent molecular weight was larger than expected. Further study showed that the fusion protein was glycosylated in CHO-S cells. The deglycosylated fusion protein ran in the expected size range in SDS–PAGE (Fig. 3). N-terminal sequence analysis of rhIFN-ω-Fc showed the following: CDLPQNHGLLSRNTL (data not shown); the signal peptide with amino acid sequences of MALLFPLLAALVMTSYSPVGSLG was excised correctly by the protease demonstrating that the N-terminal sequence of purified rhIFN-ω-Fc was correct.

3.3. Antiviral activity of rhIFN-ω-Fc

The antiviral activity of the purified rhIFN-ω-Fc was assessed by inhibition of the cytopathic effects (CPEs) of VSV virus on WISH cells. The recombinant IFN-ω (yeast *Pichia pastoris*-derived) was used for comparison. The specific activity of the purified rhIFN-ω-Fc was 1.6×10^7 IU/mg. If the specific activity of the rhIFN-ω-Fc is used to calculate rhIFN-ω according to molecular weight, the specific activity of the rhIFN-ω is 3.7×10^7 IU/mg. The purified IFN-ω (yeast *P. pastoris* derived) was 7×10^7 IU/mg. Therefore, the fusion expression can maintain good antiviral activity.

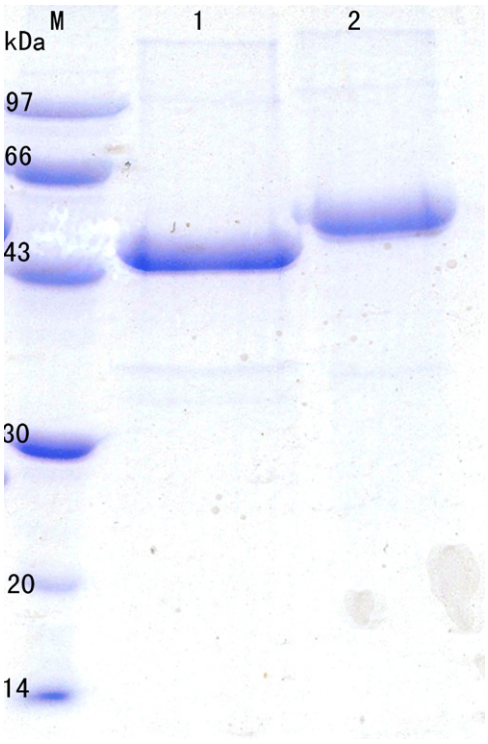

Fig. 3. Separation of rhIFN-ω-Fc by SDS–PAGE followed by Coomassie brilliant blue staining. Lane M, low molecular weight marker; lane 1, reduced and deglycosylated rhIFN-ω-Fc; lane 2, reduced rhIFN-Fc.

3.4. Pharmacokinetics

Mean plasma concentrations (±S.E.M.) of rhIFN-ω-Fc and rhIFN-ω, after a single subcutaneous dose of 1 nmol/kg, are shown in Fig. 4. Drug presence was determined in all animals through 144 h. Pharmacokinetic analysis is provided in Table 2. The absorption of the rhIFN-ω-Fc was relatively slow: the time of peak concentration was 8 h for the rhIFN-ω-Fc and 0.5 h for the rhIFN-ω. The clearance of rhIFN-ω-Fc is lower than that of rhIFN-ω. A markedly improved clearance half-life of rhIFN-ω-Fc was observed. The elimination half-life of rhIFN-ω-Fc was approximately 53.3 h whereas the elimination half-life of the rhIFN-ω was only 1.54 h. At effective therapeutic doses, the rhIFN-ω-Fc has approximately 4-fold greater C_{max} (maximal serum concentration).

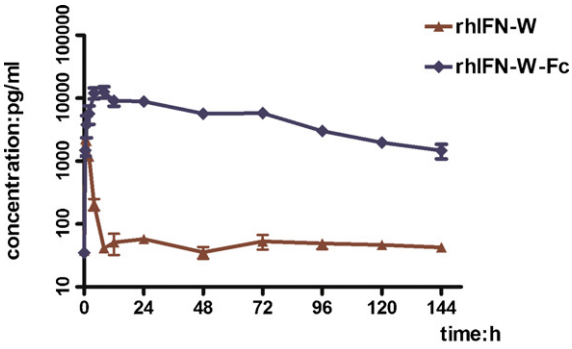

Fig. 4. Equimolar doses of the rhIFN-ω-Fc (46 μg/kg) and rhIFN-ω (20 μg/kg) were administered to rabbits by subcutaneous injections. Blood was drawn at selected times after administration. Serum concentrations of the two molecules were determined by hIFN-ω specific ELISA.

Table 2
Pharmacokinetics parameters for rabbits given a single subcutaneous of rhIFN-ω or rhIFN-ω-Fc.

Species	No. and sex of animals	Dose (μg/kg)	Dose (nmol/kg)	T_{max} (h)	C_{max} (μg/ml)	$t_{1/2}$ (h)
rhIFN-ω	5M	20	1	0.5	3025	1.54
rhIFN-ω-Fc	5M	46	1	8	12,270	53.3

4. Discussion

Interferons play increasingly important roles for the treatment of chronic viral infection, while facing challenges due to the relatively short half-life. At present, there are several effective tools for improving the half-life. PEGylated interferons have been approved for the treatment of HCV infection, having displayed the economic benefits and valid clinical compliance because of fewer injections. In addition to PEGylation, the pharmacokinetics can also be improved by constructing HSA and Fc fusion proteins.

Because hIFN-ω has glycosylation site, and the glycosylated hIFN-ω was also shown to be more potent for antiviral activity (Buckwold et al., 2007), the mammalian system was the best choice for glycoprotein expression because of the accurate glycosylation. As a result, the rhIFN-ω-Fc was expressed at high levels in CHO-S cells with a yield of 85 mg/l from spinner suspension flasks. After one-step affinity purification, rhIFN-ω-Fc expression protein can be purified up to 95%.

The specific activity of purified rhIFN-ω-Fc was 1.6×10^7 IU/mg. The molecular weight of purified rhIFN-ω-Fc is 2.3 times greater than purified rhIFN-ω. Thus, the deduced specific activity is 3.7×10^7 IU/mg of rhIFN-ω. The specific activity of the PEGASYS and PegIntron® were 1.4×10^7 IU/mg and 7×10^7 IU/mg, respectively. By comparison, rhIFN-ω-Fc has similar antiviral activity.

In the present paper, we prepared the rhIFN-ω-Fc fusion protein to investigate an alternative method of improving the serum half-life. The molecular weight of the fusion protein is 2.3 times higher than that of rhIFN-ω. The fusion protein existed in a homodimer pattern with a molecular weight of 100 kDa because inter-disulfide bonds were developed. IFN-ω-Fc was secreted from the CHO-S cell in a glycosylated pattern, further verified by PNGase digestion (Fig. 3). Both correct glycosylation and larger molecular weight will prolong the half-life in the mammalian body. For pharmacokinetics analysis, equimolar amounts of both rhIFN-ω and rhIFN-ω-Fc were injected into rabbits. The rhIFN-ω serum concentration was determined and further evaluated using GraphPad Prism 5.0 software. The rhIFN-ω-Fc fusion protein exhibited better pharmacokinetics characteristics.

In basic terms, rhIFN-ω-Fc fusion protein has a similar half-life and antiviral activity compared to the commercial products currently in use, PEGASYS and PegIntron®. In addition, rhIFN-ω-Fc has its advantages with the simple process of correct glycosylation. The results provided show promise for the development of a new alternative antiviral drug for the treatment of chronic viral infection.

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